EXHIBIT A

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Surface antigenic profile and globin phenotype of two new human erythroleukemia lines: characterization and interpretations

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Surface Antigenic Profile and Globin Phenotype of Two New Human Erythroleukemia Lines: Characterization and Interpretations

By Thalia Papayannopoulou, Betty Nakamoto, Sumiko Kurachi, Mary Tweeddale, and Hans Messner

Detailed characterization of the composite phenotype of two newly established erythroleukemia lines (OCIM1, OCIM2) shows that these lines share many of their erythroid markers (ie, surface antigens and globin program) as well as several of their nonerythroid properties (myeloid/monocytic/megakaryocytic) with the two known erythroleukemia lines (K562, HEL). In addition, each displays novel and instructive features. We argue that the surface and globin phenotype of all erythroleukemia lines is nonrandom and that it may be of physiologic relevance: it could represent the most prevalent phenotype of cells transformed by leukemia in vivo, and it raises the possibility that cells with similar potentials exist transiently during normal hematopoietic differentiation before their irreversible commitment to a single lineage. As such, these cells demonstrate a greater phenotypic adaptability in vitro than do their single lineage–committed counterparts since they can differentiate toward more than one lineage.

HEMATOPOIETIC leukemic cell lines have been considered to represent cells early in their differentiation pathway and to display several properties presumed to be present in normal hematopoietic stem cells.[1-3] Although the faithful representation by the leukemic lines of normal progenitor properties has been questioned,[4] their exploitation for the creation of immunologic, biochemical, and molecular probes valuable in the inquiry of normal differentiation processes is of undeniable utility. Numerous human leukemic lines of lymphoid or myeloid origin have been described to date, but only very few human erythroleukemia lines[5-8] have been reported thus far. The latter represent valuable cellular models for studying aspects of globin gene regulation and erythroid cell differentiation. We have recently adapted to continuous cell culture two new human erythroleukemia lines[9] and wish to present data on the characterization of these lines. By comparing them with the previous erythroleukemia lines, we point out both their common and their novel features and attempt to interpret their composite phenotype.

MATERIALS AND METHODS

Establishment of the two erythroleukemia lines. OCIM1 cells were originally derived from the leukemic blasts of a 62-year-old patient who developed erythroleukemia following a 7-year chlorambucil treatment for his chronic lymphocytic leukemia. These cells have been in continuous culture for approximately 3½ years. OCIM2 cells have been in continuous culture for approximately 3 years. They were derived from a 56-year-old patient with erythroleukemia, which represented the end stage of a previously identified myelodysplastic syndrome. Leukemic cells from these two patients were initially cloned in methylcellulose media. Subcloning experiments were repeatedly performed, and healthy methylcellulose clones, after serial passages in semisolid media, were adapted to suspension cultures. Both lines are currently maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 46 μmol/L 2-mercaptoethanol, and antibiotics.

Benzidine staining. The presence of heme or hemoglobin in these cells was evaluated by benzidine staining in either cell suspension[10] or in fixed cytocentrifuge smears.[11]

Hemoglobin analysis. Quantitative measurements of hemoglobin were done in lysates of induced and uninduced cells by a spectrophotometric method.[12] Identification of particular hemoglobin species was carried out by isoelectric focusing in polyacrylamide gels as previously described.[13] Hemoglobin bands in these gels were visualized by benzidine staining. To determine the identity of the stained hemoglobin bands, individual bands from hemoglobin gels were spliced and run in denatured NP-40 urea gels to identify their constituent globin chains.

Globin chain analysis. To separate globin chains from the lysates of induced or uninduced cells, ^{3}H-leucine–labeled cells were subjected to isoelectric focusing according to previously described methodology.[14] Before running, globin was purified through binding to immobilized haptoglobin.[15] After isofocusing, the gels were fixed, treated with En^{3}Hance (DuPont, Boston), dried, and subjected to fluorography and densitometry as previously described.[16]

Immunofluorescence with antiglobin chain antibodies. Cytocentrifuged smears of uninduced or induced cells were fixed in methanol and reacted with antiglobin chain antibodies[17] in an indirect immunofluorescence assay using antimouse IgG $(Fab')_2$ conjugated to fluorescein isothiocyanate (FITC). Two monoclonal antibodies (MoAbs), one specific for γ chains, the other for βδ chains, were used as well as a monospecific polyclonal antibody against ζ chains, kindly provided by Dr D. Chui.[18]

Surface immunofluorescence labeling. To study the surface antigen profile of these two leukemic lines, several previously characterized antibodies specific for either myelo/monocytic cells, lymphoid cells, megakaryocytic cells, or erythroid cells were used. Evaluation of labeled cells was done in a fluorescent microscope and by fluorometric analysis in a fluorescence-activated cell sorter (FACS; Ortho Diagnostics system, Westwood, MA). The antibodies used, their specificities, and sources[19-47] are displayed in Tables 1 through 4.

Allostimulation. Peripheral blood mononuclear cells (PBMC) were purified from diluted, heparinized blood by centrifugation over Lymphoprep (Nygaard & Co, Oslo) for 30 minutes at 400 g. Interface cells were washed three times and resuspended in RPMI 1640, 25 mmol/L HEPES, 20% heat-inactivated pooled human male serum, 10 IU/mL heparin, 50 IU/mL penicillin, and 50 μg/mL streptomycin.

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Table 1. Antigens Used

Surface Antigens	Reference	OCIM1	OCIM2	HEL	K562
Myeloid/monocytic					
My-1 (1G10, CD15)	19	–	–	–	++
My-7 (CD13)	20	+	+	++	++
My-9, L4F3 (CD33)	20, 21	++	++	++	++
My-10, 12.8 (BI 3C5, CD34)	22, 23	+	++	±	–
5F-1 (20.3, CD36)	24	++	++	++	–
Mac 120	25	–	+	±	±
60.3 (CDw18)	26	–	++	±	ND
Platelet/megakaryocytic					
PGP-IIb/IIIa (AP-2, 13.1)	27, 28	±	++	+++	+
PGP-Ib, (rabbit anti-PGP-Ib, C7E10)	29	–	–	+	–
10-76-3	30	–	+	+	
Thrombospondin	*	–	±	±	–
von Willebrand factor	31	–	±	±	–
Lymphoid					
Campath I	32	–	–	–	–
3A1 (CD7)	33	–	–	–	–
T1, T3, T4, T6, T8, T10, T11	†	–	–	–	–
T-200	‡	–	–	–	–
26.2 (CALLA, J5, CD10)	34	–	–	–	–
Anti-IgG	–	–	–	–	–
B4	35	–	–	–	–

Pluses indicate the degree of positivity as judged by immunofluorescence intensity (±, <1%; +, 1% to 20%; ++, 30% to 50%; +++, 50% to 100%).

Abbreviation: ND, not determined.

*Kindly donated by Dr Bornstein, Seattle.

†Ortho Diagnostic Systems, Raritan, NJ.

‡Becton Dickinson Immunocytometry Systems, Mountain View, CA.

For primary proliferation assays, 5×10^4 PBMC were cultured for six days with irradiated (1,500 to 3,700 R) stimulators in 96-well round-bottomed plates. All cultures were incubated in humidified 5% CO_2 and pulsed with 1 μCi-tritiated thymidine (New England Nuclear, Boston) for four hours before harvesting in a PHD machine (Cambridge Technology, Watertown, MA). Incorporated radioactivity was measured with a Packard liquid scintillation counter (Packard Instrument Co, Downers Grove, IL). Data represent means ± SE of triplicate cultures. In some experiments, purified human interleukin-2 (Genzyme, Boston) supernatants from concanavalin A– or allo activated PBMC or B-lymphoid line culture supernatants were added as a source of a "second" signal so that the absence of proliferation was not due to missing lymphokine(s).

Cytochemical staining. Induced and uninduced cells from the two lines were stained for PAS, myeloperoxidase, nonspecific esterase (alphanaphthol butyrase), and chloroacetate esterase as described.[49]

Cloning experiments. Cells from two leukemic cell lines were cloned in methylcellulose media, and single clones were transferred to secondary plates and subcloned at least twice to secure the single-cell origin of the clones. Individual clones, if desired, were expanded in suspension cultures and induced with several inducers to evaluate globin expression or benzidine positivity, and they were compared with parental cells.

Induction regimen. Several inducers of erythroleukemic cell lines were studied on the basis of previous information with murine and human erythroleukemic cells.[49] The various inducers, at predefined concentrations void of excessive toxicity, were added to cells at logarithmic-phase growth, and their effects on globin and heme syathesis were tested from one to five days in the presence of the inducer. Apart from the globin or hemoglobin inducers, both lines were treated with phorbol myristate acetate (PMA), and its effects were studied two days following its addition. Changes in adherence,

Table 2. Expression of HLA Antigens in Erythroleukemia Lines

HLA-Antigens	Reference	OCIM1	OCIM2	HEL	K562
B0.5 (HLA-ABC)	36	+++	+++	+++	–
4.1 (HLA-DR)	36	+++	–	+	–
B7/21 (HLA-DP)*	37	++	–	+	–
33.1, (HLA-DQ)*	38	++	–	–	–

*Experiments were performed by Gayle C. Baldwein in Dr B. Torok-Storb's laboratory. Data kindly provided to us by Dr B. Torok-Storb.

Table 3. Stimulating Potential of OCIM1 in Primary Mixed Lymphocyte Reaction

Responders	Stimulators	Experiment 1 (5:1)*	Experiment 2 (5:1)	Experiment 3 (3:1)
PBMC-A†	none	3,836	695	5,596
PBMC-A	PBMC-Ax	8,903	1,352	6,094
PBMC-A	PBMC-Bx	47,413	28,980	46,771
PBMC-A	OCIM 1x	39,478	13,801	25,004
None	none	169	55	80
None	PMBC-Ax	84	27	132
None	PBMC-Bx	138	60	123
None	OCIM 1x	2,753	304	1,184

Experiments were performed by Gayle C. Baldwein in Dr B. Torok-Storb's laboratory. Data kindly provided to us by Dr B. Torok-Storb.

*Responder to stimulator ratio (5×10^4 responders to 1×10^4 irradiated stimulators).

†PBMC-A, PBMCs from individual (A or B) mismatched for all HLA antigens.

Table 4. Erythroid Surface Antigen Profile

Erythroid Surface Antigens	Reference	OCIM1	OCIM2	HEL	K562
Glycophorin A					
Rabbit anti-glycophorin A	39	+++	++	+++	+++
10F7MN	40	++	+	++	++
R10	41	+++	+	+++	+++
F-11	29	+	+	+	+
Blood group antigens					
A		−	−	−	−
B		−	−	−	−
H (*Ulex Europaeus* I)	29	+++	−	+++	−
BE2	42	+	−	++	−
Le^a (SSEA-1)	43	−	−	−	++
i (Den, 1:2,000)		+	++	++	++
I (Ma, 1:1,000)		+++	±	±	+
Other					
Ep-1	44	++	++	+	+
Ep-2	44	++	++	±	+
69.20	45	++	+	+	+
69.15	45	+	+	+	+
69.23	45	++	++	++	++
80.14	45	+	+	+	+
L5-1	46	+++	++	+	+
SH.23.6	47	+	+	+	+

proliferative potential, and surface antigen expression were studied. Methodologic details for these studies have been previously described.[31,50]

Preparation of RNA and S1 nuclease analysis. Total cellular RNA was prepared by cell lysis in 4 mol/L guanidine hydrochloride; this was followed by cesium chloride gradient (2 g/mL) centrifugation at 32,000 g for 40 hours. The RNA pellet was dissolved in 2 mL of buffer containing 100 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5, 1.0 mmol/L EDTA, and 1.0% sodium dodecyl sulfate and, following phenol extraction, was twice precipitated in ethanol and stored at −70°C. After centrifugation, the RNA was dissolved in water and was used for S1 nuclease analysis. A uniformly labeled M13mp7 probe specific for β- and γ-globin RNA and hybridization conditions were as described in detail previously.[51,52]

RESULTS

Growth requirements and clonogenic potential of the two erythroleukemia lines. Primary leukemic cells from the two patients were initially grown in clonal methylcellulose cultures. During this time, there was an absolute dependency on the use of prescreened human plasma (either aplastic plasma or, rarely, normal plasma) for successful growth. The presence of 30% human aplastic plasma not only afforded the highest plating efficiency (~30%), but the colonies were larger, and several of them acquired a reddish color and hemoglobinization. The addition of exogenous erythropoietin (Epo; up to 10 U/mL) or other growth factors in the form of conditioned media from the Mo cell line or phytohemagglutinin-lymphocyte-conditioned medium increased neither the size nor the number of colonies over that observed in aplastic plasma. Healthy methylcellulose clones were repeatedly propagated in methylcellulose media, and multiple attempts were made to lift methylcellulose clones and expand them in suspension culture. This was not successful initially. However, after repeated replatings and clonal selection it was finally successful. Cells in suspension were initially maintained in the same media as in clonal cultures. Later, however, these cells were gradually adapted to the presence of normal human serum or fetal calf serum. Cells in suspension culture reach a saturation density of 2×10^6/mL and require a change of media about twice weekly. No significant differences between the two lines in cloning efficiencies (26% and 30%, respectively) in semisolid media were observed initially, and both were dependent on prescreened human plasma. However, OCIM1 was distinguished by the presence of several hemoglobinized colonies that were absent in OCIM2. Hemoglobinized (red) colonies from OCIM1 were propagated separately from colonies nonhemoglobinized (white) in this cell line, and they are called OCIM1 and OCIM1-R hereafter.

Morphology and cytochemical characteristics. The appearance of OCIM1 cells in standard smears is quite characteristic because of their large size (average size, >16 μm), their nuclear morphology (large nuclei with sharply stained nuclear membrane), and a tendency to form multinucleated large cells. OCIM2 cells, on the other hand, are much smaller (~11 μm) and have intense basophilic cytoplasm, large nuclei, and a larger nuclear/cytoplasmic ratio. The cytochemical characteristics are summarized as follows: both OCIM1 and OCIM2 cells are peroxidase- and chloroacetate esterase–negative, but they are virtually all PAS-positive and nonspecific esterase (alphanaphthol butyrase)-positive. The cytochemical properties of the two cell lines reflected, by and large, those of the leukemic blast cells of the patients (data not shown). Erythroid induction did not basically change these cytochemical features; however, reduction (by 40%) in PAS positivity was observed in both lines postinduction.

Surface antigenic phenotype of OCIM1 and OCIM2. The surface antigens expressed by OCIM1 and OCIM2 cells were studied through the use of MoAbs or polyclonal antibodies with specificities against cells of lymphoid, myeloid, platelet/megakaryocytic, or erythroid lin-

Table 5. Effect of PMA on Surface Antigen Expression by OCIM1 and OCIM2 Cells

Cell Type	Treatment	Antigens (Percent Expression) DR (4.1)	PGPIIb/IIIa (13.1)	PGP-Ib*	DQ (33.1)
OCIM1-parent	none	83	1	<1	80
	PMA†	86	13	2	11
OCIM1-R	None	44	1	0	ND
	PMA	22	7	0	ND
OCIM2-parent	None	0	10	0	0
	PMA	0	47	0	0
OCIM2-R	None	0	14	0	0
	PMA	0	40	0	0

*Polyclonal anti-PGP-Ib antibody was kindly provided by Dr G. Roth, Seattle.

†PMA at 1.6×10^{-7} mol/L for 48 hours.

cage (Tables 1, 2, 4, 5). Antigens present on more mature granulocytic cells and detected by MoAbs 1G10 or My-1 are virtually absent in the two leukemic lines (Table 1). However, antibodies recognizing determinants present in immature myeloid cells (My-7, My-9, My-10) are reactive with these two cells lines (Fig 1A). Reactivity with My-10 is of interest since only KG1a cells were previously reported as positive.[22] Regarding the megakaryocytic series, one polyclonal antibody and three MoAbs against the glycoprotein IIb/IIIa and one polyclonal antibody and two MoABs against glycoprotein Ib were tested (Table 1). OCIM2 is strongly reactive with all anti-glycoprotein IIb/IIIa antibodies (Fig 1B), whereas OCIM1 is only weakly reactive. Weak to absent reactivity was also found with anti-PGP Ib in both cell lines. All antilymphoid antibodies tested were found to be negative in these two lines (Table 1).

Both lines are strongly positive in HLA-ABC determinants, but they differ in the expression of HLA-D region determinants (Table 2). OCIM1 cells are virtually all positive in HLA-DR and HLA-DP (Fig 2A) and about 30% to 40% positive in HLA-DQ antigens when using antibodies with previously characterized specificities (Table 2). To test whether the DQ antigen functions in alloreactivity settings, three independent experiments were set up by using several combinations of stimulator and responder populations from peripheral blood as described previously in similar experiments with HEL cells.[54] From the data in Table 3, it is apparent that OCIM1 cells can elicit alloreactivity responses since they stimulate the proliferation of mismatched lymphocytes.

The pattern of reactivity with antierythroid monoclonal antibodies was studied in detail (Table 4). The majority of cells, especially in OCIM1 and less so in OCIM2, are positive for antiglycophorin MoAbs. Both lines are also widely reactive with the antierythroid antibodies Ep-1 and Ep-2[44] and partially reactive with a MoAb (SFL 23.6) against an antigen present in the late, differentiated erythroid series.[47] In addition, the lines react significantly with a group of

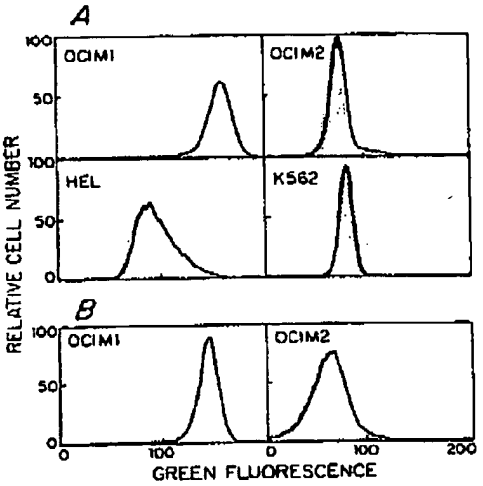

Fig 2. (A) Fluorescent profiles of OCIM1, OCIM2, HEL, and K562 cells after labeling with an anti-HLA-DR framework antibody (4.1). OCIM1 appears to be strongly reactive with this antibody, while HEL cells are partially reactive, and OCIM2 and K562 are nonreactive. (B) Profiles of OCIM1 and OCIM2 cells labeled with the lectin *Ulex Europaeus* I (binding antigen H) conjugated to FITC. A strong reactivity with OCIM1 but not OCIM2 is noted.

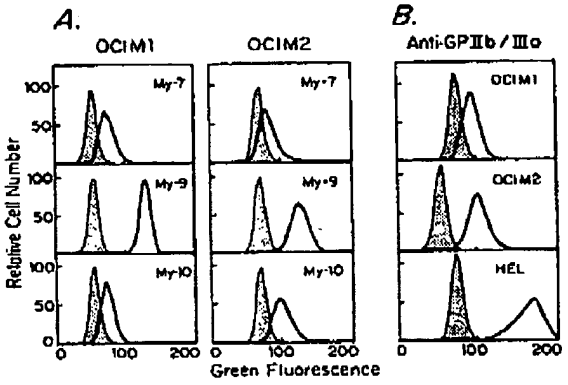

Fig 1. FACS profiles of OCIM1 and OCIM2 after labeling with MoAbs My-7, My-9, My-10. (A) anti-GPIIb/IIIa (B) followed by IgG $(Fab')_2$-FITC. Shaded areas are profiles of the same cells labeled with an irrelevant antibody of the same isotype and the same second antibody. (A) Significant reactivity is observed with antibodies reacting with early myeloid cells, ie, My-10, My-9, My-7, but virtually no reactivity was found with My-1 or 1G10, ie, antigens present on more mature myeloid cells (Table 1). (B) Reactivities of OCIM1 and OCIM2 cells with anti-glycoprotein IIb/IIIa (AP-2) are compared with those of HEL cells labeled with the same antibody.

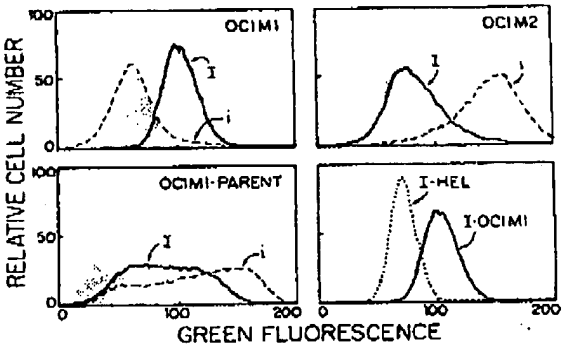

Fig 3. Fluorescent profiles of OCIM1 and OCIM2 cells labeled with anti-I (anti-i Den, 1:2,000 dilution) and anti-I (anti-I Ma, 1:1,000 dilution) and antihuman IgM-FITC. The stronger reactivity of OCIM1 cells against anti-I as compared with anti-i is evident. However, parental OCIM1 cells are equally reactive with both antibodies (lower left panel). The pattern is reversed in OCIM2 cells in which reactivity with anti-i predominates (upper right panel). The panel in the lower right compares the reactivities of OCIM1 and HEL against anti-I: HEL cells are negative in I expression.

Table 6. OCIM1

Inducer	Immunofluorescence: Benzidine+ (%)	γ-Globin+ (%)	δβ-Globin+ (%)	ζ-Globin+ (%)	Viability (%)
OCIM1-parent					
None	0	10	0	0	95-100
δ-ALA (500 μmol/L)	<1	12	Rare	0	90
OCIM1-R					
None	3-12*	20-45	0	0	88-100
Hemin (50 μmol/L)	16	52	0	0	83
δ-ALA (500 μmol/L)	15-60	50-82	<0.1	0	90-97
Ara-C (0.36 μmol/L)	17	69	5	0	72
Na butyrate (1.0 mmol/L)	26	63	0	0	72
5-Aza (20 μmol/L)	4	33	0	0	34

Abbreviations: Ara-C, cytosine arabinoside; 5-Aza, 5-azacytidine.
*Ranges indicate data from multiple experiments.

MoAbs positive against normal erythroid cells.[45] Regarding blood group antigens, there is strong reactivity against anti-H and anti-I in the OCIM1 cells (Figs 2B and 3). Anti-I reactivity exceeds the anti-i reactivity, thus presenting a pattern characteristic of the adult erythroid cells. OCIM2 is i-positive and has only a minor population reacting with anti-I, thus resembling K562 cells in its pattern of il reactivity; HEL cells are virtually negative for I antigen, but they are all positive for i antigen (data not shown).

Expression of heme and globin. There was a very low proportion of benzidine-positive cells (0.0% to 0.5%) in parental cells from both lines, but significant benzidine positivity (1% to 4%) was found in selected populations (eg, OCIM1-R) before induction (Tables 6 and 7). Benzidine positivity in fixed preparations of OCIM1 cells showed a somewhat unusual pattern in that the nuclei were strongly positive, often more so than the cytoplasm (data not shown). Determination of hemoglobin content showed that noninduced OCIM1 cells contained, on the average, 0.1 pg hemoglobin/cell, while much less than that was found in OCIM2 (~0.02 pg/cell). Several erythroid inducers previously found to be effective in other erythroleukemia lines (MEL, K562, HEL) were tried with these two lines (Tables 6 and 7). Best induction of both heme and globin was afforded by the addition of δ-aminolevulinic acid (δ-ALA), which increased the hemoglobin per cell up to ten times. Not only was efficient induction achieved, but the viability of cells and initial proliferation in both lines were not affected by this inducer. Cell lysates from δ-ALA–induced cell lines were subjected to isoelectric focusing in polyacrylamide gels followed by benzidine staining to identify the hemoglobin species present in these cell lysates. As seen in Fig 4, OCIM2 produces mainly HbF ($\alpha_2\gamma_2$), Hb Bart's (γ_4), and Hb Portland ($\zeta_2\gamma_2$); OCIM1 cells produce predominantly HbF and Hb Bart's. In addition, a band in the position of HbA is notable. Subsequent analysis, however, of the globin chains present in this Hb band disclosed the presence of modified γ chains (acetylated) rather than β chains (data not shown). Furthermore, when whole-cell lysates were run under denatured conditions in the presence of NP-40–urea, no β chains were present in both cell lines, whereas there was an abundance of γ chains, α chains, and in OCIM2, ζ chains and δ chains (Fig 5, Fig 6, left panel).

The presence of globin was also tested at a cellular level

Table 7. OCIM2

Inducer	Immunofluorescence: Benzidine+ (%)	γ-Globin+ (%)	δβ-Globin+ (%)	ζ-Globin+ (%)	Viability (%)
OCIM2-Parent					
None	0-0.5*	17-23	1-4	5-12	97-100
Hemin (60 μmol/L)	9	30-60	3-9	14-17	91
δ-ALA (500 μmol/L)	6-14	35-62	4-19	19-27	92
Ara-C (.36 μmol/L)	0	31	20	25	42
(.002 μmol/L)	0	25	7	16	—
Na Butyrate (1.0 mmol/L)	8-11	33-45	5-12	24	78
5-Aza (20 μmol/L)	0	5	0	2.5	54
OCIM2-R					
None	0.1-4	24-49	2-10	19	>90
δ-ALA	41	43-80	3-8	53	7
Na butyrate (0.5 mmol/L)	19	25	3	ND	72
BrDU (32.5 μmol/L)	9.5	27	12	ND	76

Abbreviation: BrDU, bromodeoxyuridine.
*Ranges indicate data from more than one experiment.

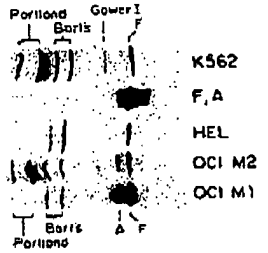

Fig 4. Hemoglobin isoelectric focusing of cell lysates from δ-ALA–induced OCIM1 and OCIM2 cells. The gels were stained with benzidine to identify hemoglobin bands. As controls, a mixture of HbA and HbF was used as well as induced K562 cells. Definitive identification of major hemoglobin bands in OCIM1 and OCIM2 was done by cutting the individual bands and subjecting them to isoelectric focusing in NP-40–urea gels to separate their constituent chains.

through the use of fluorescent antiglobin chain MoAbs (Tables 6 and 7). Before induction, a significant number of cells were positive with anti–γ-globin MoAbs (up to 45% in OCIM1 and up to 25% in OCIM2 cells). OCIM1 cells were negative in anti–βδ- and anti–ζ-globin antibodies; however, OCIM2 cells had positive cells with both of these antibodies, and positivity increased further postinduction (Table 7). Since β chains were virtually absent by isoelectric focusing in OCIM2 cells and the antibody used reacts with both β and δ chains, the positivity seen with the antibody is attributed to the presence of δ chains. This was further verified by S1 nuclease analysis using β- and δ-specific probes (Fig 6). Although significant levels of δ-mRNA were found in parental OCIM2 cells and the great majority of its subclones, β-mRNA was not detected (Fig 6, middle panel).

In addition to single immunofluorescence labeling (with

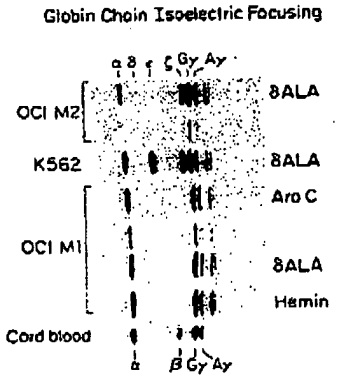

Fig 5. Lysates from ^{3}H-leucine–labeled OCIM1 and OCIM2 cells were subjected to isoelectric focusing in NP-40–urea gels before and after induction. Labeled control lysates were from K562 cells and from cord blood reticulocytes. OCIM1 cells produce almost exclusively γ ($^{G}\gamma > {}^{A}\gamma$) and α chains, whereas OCIM2 cells, in addition to γ and α chains, synthesize ζ chains and traces of ε and δ chains.

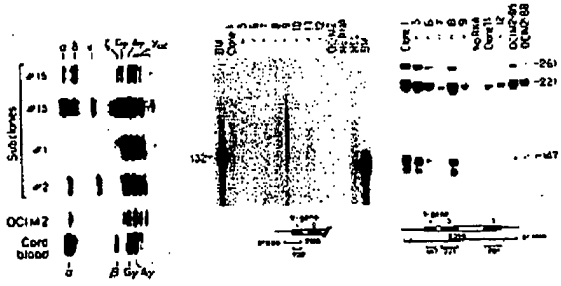

Fig 6. Globin expression in OCIM2 subclones. (Left panel) Isoelectric focusing of globin chains of OCIM2 parental cells and of four select subclones. All subclones produce γ chains; however, extreme variations in relative proportions of δ (clone 1), ε (clones 2 and 13), and ζ (clone 15) are present. (Middle and right panel) S1 nuclease analysis of β- and δ-globin gene expression in 9 OCIM2 subclones (BM, bone marrow, 2 μg total cellular RNA; clones, 20 μg). When a β-specific probe was used, no β-mRNA was detected (middle panel). By contrast, significant levels of δ-mRNA were detected (right panel) in the majority of the subclones.

anti-βδ or anti-γ), double-labeling experiments were done after induction (ie, either anti-γ-FITC followed by anti-βδ–rhodamine or anti-ζ+ antirabbit IgG-FITC followed by βδ-rhodamine). In OCIM2, the expression of adult (βδ) globin was, by and large, cellularly segregated from γ- (Fig 7) or ζ-globin (data not shown). To test whether the different cells in OCIM2 had distinct, stable, and heritable patterns of globin expression, we subcloned the original population. A total of 15 subclones were analyzed by antiglobin immunofluorescence, nine by S1 nuclease analysis, and select ones by globin chain isoelectric focusing. All subclones contained

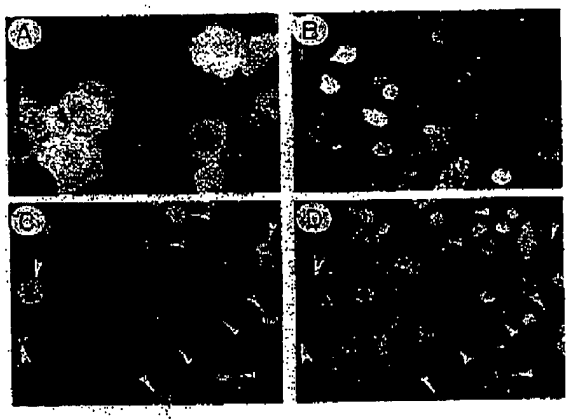

Fig 7. Immunofluorescence labeling of induced OCIM1 and OCIM2 cells with antiglobin chain antibodies. (A) Labeling of OCIM1 cells by anti-γ-FITC. Note an unusual prominence in nuclear staining. (Nuclei also stain positive with benzidine; see the text). (B) Labeling of induced (δ-ALA) OCIM2 cells with anti-ζ, and antirabbit IgG–FITC. About 25% positive cells are seen. (C and D) Double labeling of OCIM2 cells with anti-βδ–rhodamine (C) and anti-γ–FITC (D). Bright, βδ-positive cells in C (shown by arrows) can be traced in D (arrows in the same cells as C), and they are largely unlabeled by anti-γ.

γ-globin and in various proportions the other globin species (Table 8). Extreme variations included the virtual absence of α chains or high levels of δ or ε chains in some subclones (Fig 6, left panel). Distinct, exclusive globin patterns (ie, only adult, only fetal, or only fetal and embryonic) were not found.

Treatment with PMA. Treatment with phorbol esters at doses from 10^{-9} through 10^{-6} mol/L PMA induced changes that were very similar to the ones previously seen in K562 or HEL cells.[31,50] For example, following treatment of OCIM2 with PMA for at least 24 hours, the majority of the cells adhere to plastic surface with subsequent spreading (over 80% of OCIM2 cells became adherent but only rare OCIM1 cells following PMA [1.6×10^{-7} mol/L] treatment). Constitutive levels of globin expression (assessed by the proportion of γ-globin–positive cells) were decreased in both cell lines after treatment, and the cells became resistant to subsequent globin induction by an inducer. Specific changes in surface antigen expression (Table 5) included enhancement of GPIIb/IIIa expression, some augmentation in GPIb reactivity when a polyclonal antibody was used, and a relative decrease in HLA-DR expression in OCIM1 cells.

Phenotypic changes over time. Since the initial characterization of the OCIM1 and OCIM2 cells during the first year of their establishment, a few changes have been observed after 2 additional years of culture. In OCIM1, a progressive decrease in the number of cells with constitutive and inducible expression of hemoglobin has become apparent. As a result the cells produce fewer red colonies in the clonogenic assays, and there is a reduction in hemoglobin accumulation (red color) following induction. However, it has been possible to select reddish colonies and maintain them by subcloning a population similar to the one originally selected. On the other hand, OCIM2 was initially characterized by severe heme deficiency, as indicated by the very low number of benzidine-positive cells but a high number of globin cells following induction. However, following persistent efforts, it has been possible to isolate populations that become visibly hemoglobinized upon induction. Recent data show up to 41% benzidine-positive cells postinduction in these highly inducible cells (OCIM2-R, Table 7). Thus, the ability to accumulate hemoglobin in these two lines as well as in HEL cells has not been a stable property.

Table 8. OCIM2: Globin Expression in 15 Induced (δ-ALA) Subclones

Subclone No.	Benzidine+ (%)	γ+ (%)	βδ+ (%)
1	23	51	18
2	1	41	6
3	0	14	9
4	0	22	11
5	2	25	21
6	8	32	19
7	0	6	15
8	1	17	40
9	0	25	7
10	<1	3	15
11	7	42	7
12	6	11	44
13	13	47	21
14	5	29	9
15	2	17	25
OCIM2-parental	2	35	19
OCIM2-R	41	60	3

DISCUSSION

All the erythroleukemic lines described thus far, including the ones in the present report, appear to display a gradation of properties present within the erythroid lineage from the early progenitor stage (ie, HLA-DR or My-10 antigen expression) down to terminally differentiated cells (ie, presence of glycophorin, hemoglobin, etc). Such a combination of "early-only" and "late-only" differentiation markers deviates from the normal intralineage differentiation sequence and highlights the failure to complete the differentiation process that appears to be the hallmark of the leukemias. In addition to the common properties some of the lines have distinct features. The OCIM1 line is of particular interest for its unique combination of surface antigens. For example, the il antigenic determinants are displayed with a normal adult ratio, ie, predominance of I ν i expression. This pattern contrasts with the virtually exclusive presence of i in all other lines (K562, HEL, OCIM2, LAMA-84) and renders OCIM1 a valuable cellular model in studying the branching enzyme responsible for the conversion of i to I. It is of note, however, that despite the expression of the adult il phenotype, which is characteristic of mature adult precursors and red cells, the line does not produce adult hemoglobin. This maturational asynchrony or uncoupling of globin expression and il surface antigens, most likely consequent to their leukemic transformation, is compatible with the independent regulation of il determinants and globin type as shown previously.[52]

The expression of functional HLA-DQ antigens in OCIM1 cells is novel and possibly instructive. In contrast to DR and DP, DQ has not been found in the other erythroleukemia lines. Low levels of expression of DR and DP antigens have been found in HEL cells,[29,54] but DQ expression was absent both at the protein and mRNA level, and the cells were unable to induce allostimulation.[54] There is some ambiguity in the literature about the presence of DQ in normal hematopoietic progenitors,[55-57] and this is likely attributed to overlapping specificities of the antibodies used. Recent evidence, however, suggests that DQ is present in some CFU-GM but probably absent in BFU-E and CFU-GEMM.[58] It is thus unlikely that the presence of DQ in OCIM1 cells is part of HLA-D region expression at some stage of erythroid cell development and possibly denotes a tendency for lymphoid or, more likely, monocytic differentiation. Of note, there was no evidence of any lymphoid antigen expression (Table 1), terminal deoxynucleotidyl transferase reactivity, or T-cell receptor β chain rearrangement in these cells (data not shown). In general, the patterns of expression of HLA class II antigens in the erythroleukemic lines (ie, DR/DP in HEL and LAMA-84 and DR/DP/DQ in OCIM1) do support the view that DQ antigens are regulated independently from DR and DP as data with normal cells suggest. An additional

unique feature of OCIM1 cells is the presence of Epo receptors at levels significantly higher than in any other human line tested.[39] Although the line does not differentiate in the presence of Epo, certain aspects of Epo interaction with its receptor could be explored in this line.

Studies of globin expression in the two lines by a variety of approaches (immunofluorescence, isoelectric focusing of hemoglobin and globin chains, or studies at the mRNA level) uncover further interesting features. This comprehensive approach emphasizes that caution should be exercised in the interpretation of data when only one method is used for globin analysis (ie, hemoglobin electrophoresis or isoelectric focusing). Modified globin chains (acetylated or glycosylated) can lead to formation of hemoglobin bands with altered mobility and thus misidentification of hemoglobin species. The predominant globin expressed by both lines is the fetal. In addition, in OCIM2 there is a significant expression of δ-globin and high expression of ζ-chains (α-like embryonic chains) with less expression of ϵ chains (β-like embryonic chains). This phenotype, like the one of HEL-R,[40] underscores the lack of coordinate expression of the two types of embryonic chains (ϵ, ζ) and shows, in addition, a segregated expression of the two adult-type chains (δ and β). Moreover, when the globin expression was analyzed at the cellular level through fluorescent antibody studies, it became clear that the majority of adult globin (δ)-producing OCIM2 cells do not coexpress γ or ζ chains (Fig 7). This heterogencity in the cellular expression of globins is of interest and allows some testable predictions. If there are inherent and stable differences among cells of each line in their potential to express certain globin phenotypes, then in subcloning experiments one would expect distinct patterns in globin expression (ie, expression of either γ- or $\delta\beta$-globin.). However, subcloning studies in the present lines as well as in HEL and K562 cells[40] failed to generate segregated globin patterns and showed the same heterogeneity present in the parental cells. These data are compatible with the notion that the cells of each erythroleukemia line have a given probability (stochastic?) to express a particular set of globins and it is this potential that is inherited in the subclones rather than a stable expression of one or another globin. Although extreme variations in the relative proportion of globin chains can be generated, it is unclear how stable these patterns are (Fig 6).

In summary, the present and previous data with the erythroleukemia lines allow the following interpretative conclusions: (*a*) five of six erythroleukemia lines (K562, HEL, OCIM1, OCIM2, LAMA-84) express markers of multiple cell lineages. This may have some physiologic relevance in vivo since expression of markers from "illegitimate" lineages has been found only infrequently in primary leukemias other than erythroleukemias.[41] (*b*) They can adapt through inducers two disparate differentiative pathways in vitro (ie, erythroid with erythroid inducers or megakaryocytic/monocytic with phorbol esters), suggesting that cells at this stage, in contrast to their single-lineage counterparts, are not phenotypically rigid.[42] (*c*) They harbor an environment that allows activation of developmentally primitive globin programs (ie, fetal or embryonic) that are never manifested in fully mature normal erythroid cells. These programs may be aberrant because of leukemic transformation, or they may represent the expression of the globin potentials of normal progenitors at earlier differentiation stages before their irreversible commitment to a specific lineage. (*d*) They have instructive features that can be exploited for diagnostic purposes in covert or cryptic erythroleukemias; for example, they can be heme deficient and α chain deficient so that nonhemoglobinized cells might be a more frequent occurrence in erythroleukemia than was previously appreciated. Therefore, markers other than hemoglobin may be more sensitive in uncovering the erythroid phenotype of leukemic cells (Anderson et al,[39] Tomonaga et al,[43] and our own unpublished data). (*e*) They point to a close association of erythroid and megakaryocytic marker expression (four lines have prominent megakaryocytic markers), although both pure types of leukemias are rare. The fact that this association may be of revelance in normal differentiation is illustrated by reports that maturation of megakaryocytes is influenced by Epo[44] and that Epo increases platelet production in vivo[45] and by the recent suggestion that megakaryocytes possess Epo receptors.[46] Furthermore, in vitro it has been possible, as is the case with normal megakaryocytic and endothelial cells, to enhance after treatment with phorbol ester, their megakaryocytic phenotype and platelet-derived growth factor (PDGF)-like protein production (PDGF-A and -B by K562 and OCIM2[21,47] or PDGF-B by HEL cells.[48]) (*f*) Although none of the erythroleukemic lines responds to the physiologic regulator of erythropoiesis Epo, our experience indicates that the primary leukemic cells from which these populations were selected were sensitive to Epo.

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